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Molecular Movement inside the Translational Engine

Review

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Translation requires iterative coupled movement of mRNA and tRNA throughout the elongation phase of protein synthesis. Each new amino acid is recruited to the ribosome as an aminoacyl-tRNA·EF-Tu·GTP ternary complex. Following peptide bond formation, the tRNAs and associated mRNA must be translocated from one ribosomal site to the next in a GTP-dependent process that is catalyzed by elongation factor EF-G. On a molecular scale, this movement is substantial, involving excursions on the order of 50 Å at the elbow of tRNA during each elongation step. Although the elongation cycle requires the two G proteins (elongation factors EF-Tu and EF-G) under physiological conditions, it has been shown that protein synthesis can be carried out by the ribosome itself, in the absence of factors, or GTP, under certain in vitro conditions (Pestka, 1969; Gavrilova et al., 1976). Thus, the ability to move mRNA and tRNA is an inherent property of the ribosome; the factors serve to increase the speed and accuracy of elongation in a GTP-dependent manner. The ribosome can therefore be considered as a macromolecular machine.

Because of the fundamental importance of translation to all life as we know it, and the essential similarities between all ribosomes, the movement associated with the translational elongation cycle must be one of the most ancient and basic in biology. Understanding the underlying molecular basis of this movement has presented a formidable challenge to generations of molecular biologists. The ribosome is large (about 2.5 MDa) and structurally complex (more than 50 different proteins and three RNA molecules comprising over 4500 nucleotides; Hill et al., 1990; Matheson et al., 1995). It is also functionally complex. It is divided into a small and a large subunit, which in bacteria are called the 30S and 50S subunits. In addition to movement, the ribosome must provide specific binding sites for mRNA, tRNA, and the various initiation, elongation, and termination factors, and catalyze peptide bond formation. It must also stabilize codon–anticodon interaction and preserve the translational reading frame.

In recent years, many fundamental assumptions concerning the translational elongation cycle have been challenged, and some have required drastic revision. In this article, we focus on the process of translocation—the precisely orchestrated movement of tRNA from one ribosomal site to the next, coupled to movement of mRNA. We attempt to present a brief overview of our current understanding of the mechanism of translocation, raise or restate some basic questions, and offer some suggestions about the workings of the machine. Further discussion as well as alternative views of translocation can be found in the excellent reviews by Spirin (1985), Abel and Jurnak (1996), and Czworkowski and Moore (1996). Our point of reference will be the bacterial ribosome, from which the vast majority of our information is derived.

The Translational Elongation Cycle

We begin by placing translocation in the overall context of the translational elongation cycle. The original two-site mechanism for elongation, proposed by Watson over 30 years ago, was elaborated by the discovery of a third site, called the exit, or E site (Rheinberger et al., 1981), and many of the details of the model were confirmed or extended. The three-site version of the classical model is summarized schematically in Figure 1. Beginning with an initiator or peptidyl tRNA in the P (peptidyl) site (Figure 1A), a new aminoacyl tRNA, with an anticodon that is complementary to the available A (aminoacyl)-site codon, is introduced as an aminoacyl-tRNA·EF-Tu·GTP ternary complex. Following hydrolysis of GTP and release of EF-Tu, the aminoacyl-tRNA is bound to the A site (Figure 1B). The anticodon ends of both tRNAs interact with the 30S subunit, and their acceptor (aminoacyl) ends interact with the 50S subunit. Attack of the peptidyl-tRNA bond by the α-amino group of aminoacyl-tRNA, a spontaneous reaction catalyzed by peptidyl transferase (an activity of the 50S subunit), results in peptide bond formation and transfer of the growing peptide chain to the A-site tRNA (Figure 1C). Movement of the newly created peptidyl-tRNA from the A to P site is accomplished by EF-G in a GTP-dependent reaction (Figure 1D). At the same time, the deacylated tRNA moves to the E site. The E site is most likely located exclusively on the 50S subunit (Kirillov et al., 1983; Lill and Wintermeyer, 1987; Moazed and Noller, 1989a); this would mean that the elaborated elongation cycle is really a two-and-a-half-site, rather than a three-site, model. Deacylated tRNA, bound weakly to the E site, dissociates from the ribosome to complete the cycle of elongation. It has been proposed that binding of the next aminoacyl-tRNA to the A site allosterically weakens the affinity of tRNA for the E site (Nierhaus, 1990), but this has recently been challenged (Semenkov et al., 1996).

Chemical footprinting studies showed that tRNAs bound in their various ribosomal binding states protect characteristic bases in rRNA from chemical probes, providing structural correlates for the states of tRNA during the elongation cycle (Moazed and Noller, 1986, 1989a). It was found that, in certain intermediate states of elongation, the two ends of a tRNA could be in different states on the two ribosomal subunits; for example, a tRNA could simultaneously occupy the 30S A site and the 50S P site. Interpretation of these experiments resulted in the hybrid states model for the elongation cycle, shown in Figure 2 (Moazed and Noller, 1989b). An important implication for the mechanism of translocation was that movement of tRNA can occur independently with respect to the two ends of the tRNA relative to the two ribosomal subunits. Prior to GTP hydrolysis, aminoacyl-tRNA is bound in the A/T state (Figure 2B); i.e., its anticodon is bound to the codon in the A site of the 30S subunit, while its acceptor end is bound to a

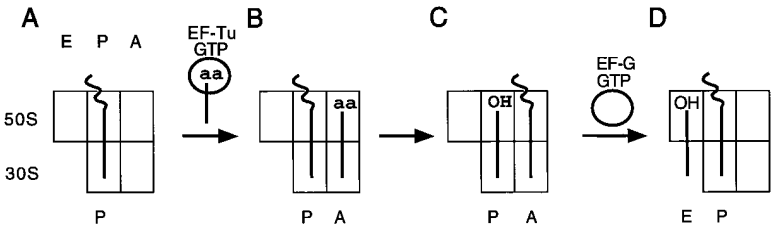

Figure 1. Classical Three-Site Model for the Translational Elongation Cycle

The tRNA binding sites on the 50S and 30S subunits, according to the three-site model (Rheinberger et al., 1981; Lill and Wintermeyer, 1987), are represented schematically by the upper and lower rectangles, respectively. The 50S subunit is subdivided into A (aminoacyl), P (peptidyl), and E (exit) sites; the 30S subunit is subdivided into A and P sites. The tRNAs are represented by vertical bars and the nascent polypeptide chain by a wavy line; aa represents the aminoacyl moiety and OH the deacylated 3′ end of tRNA; the circles represent elongation factors EF-Tu and EF-G, respectively. The binding states (A, P, and E) for the different tRNAs are indicated at the bottom of each panel.

site different from the A site of the 50S subunit, called the T site (it is probably bound mainly to EF-Tu, which is, in turn, bound to a specific site on the 50S subunit). Following GTP hydrolysis, aminoacyl-tRNA moves into the A/A state, corresponding to the classical A site (Figure 2C). Peptidyl-tRNA is bound in the P/P state, the counterpart of the classical P site, and the state of the ribosome is equivalent to that shown in Figure 1B for the classical model. Next, peptide bond formation occurs. Following peptide bond formation, both tRNAs are found in hybrid states (Figure 2D). The peptidyl-tRNA is in the A/P state, in which its anticodon is in the 30S A site, while its acceptor end is in the 50S P site. The deacylated tRNA is in the P/E state, in which its anticodon is in the 30S P site and its acceptor end in the 50S E site. Movement into the hybrid states is spontaneous, occurring independently of EF-G and GTP. Although the acceptor end of peptidyl-tRNA is in a state closely resembling binding to the 50S P site, it is not yet competent for peptide bond formation with puromycin, an aminoacyl-tRNA analog, for reasons that are not understood. Finally, EF-G catalyzes movement of the anticodon ends of both tRNAs relative to the 30S subunit, moving peptidyl-tRNA into the P/P state and deacylated tRNA into the E state (Figure 2E).

Several inferences can be made from the hybrid states

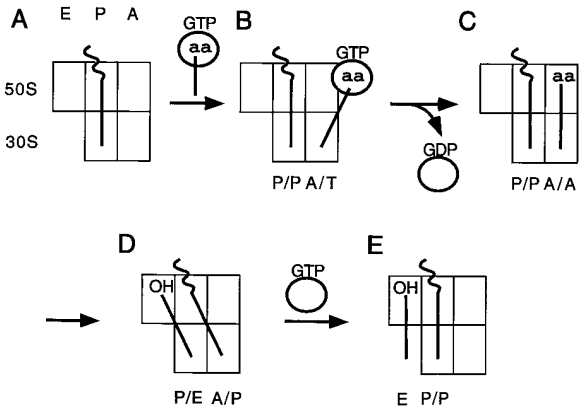

Figure 2. Hybrid States Model for the Translational Elongation Cycle

Binding states for tRNA are based on chemical footprinting studies (Moazed and Noller, 1989b). Symbols are as described for Figure 1. Binding states, indicated at the bottom of each panel, indicate the state of each tRNA relative to each ribosomal subunit; e.g., A/P indicates interaction of the anticodon end of a tRNA with the 30S A site and its acceptor end to the 50S P site.

model. (1) Translocation occurs in two steps: first, the acceptor ends of both tRNAs move relative to the 50S subunit, while the anticodon ends remain anchored to the 30S subunit; second, the anticodon ends move relative to the 30S subunit, while the acceptor ends remain anchored in the 50S subunit. (2) The first step can occur spontaneously, while the second step is an EF-G-catalyzed step requiring GTP. (3) The first (spontaneous) step provides a rationale for the E site and for its location exclusively on the 50S subunit. By virtue of its specific affinity for deacylated tRNA, the E site could provide a thermodynamic driving force for the spontaneous step of translocation. The lack of a 30S E site would be simply explained if the primary role of the E site were to establish the hybrid P/E state. (4) Independent movement of tRNA relative to the two ribosomal subunits suggests that tRNA translocation could involve relative movement of the subunits, providing an explanation for the universal two-subunit structure of ribosomes. This idea was proposed many years earlier by Bretscher (1968) and Spirin (1968). (5) The peptidyl moiety remains in the 50S subunit P site throughout the elongation cycle, rather than oscillating between the P and A sites as in the classical model.

More evidence supporting the hybrid states model has come from physical studies. Using fluorescent probes attached to specific locations on both tRNA and the ribosome, Hardesty and coworkers have measured tRNA-ribosome distances before and after peptide bond formation, by nonradiative energy transfer (Odom et al., 1990). Fluorescent probes were attached to either the 5′ end or position 8 of peptidyl-tRNA and to either protein S21 or L1. Upon peptide bond formation, the 5′ end of the peptidyl-tRNA moved at least 20 Å toward L1, and the probe attached to position 8 of the tRNA moved 10 Å toward S21. Since two of the E-site protections are in the region of 23S rRNA that interacts with protein L1, this result provides physical evidence for the P/P to P/E transition. More recently, Wintermeyer and coworkers (Borowski et al., 1996) have tested the activity of a mutant EF-G in which domain I (the catalytic or "G domain") was deleted. Using ribosomes containing a deacylated tRNA and a peptidyl-tRNA analog in the pretranslocation state, they found that the mutant factor converted the peptidyl-tRNA from a puromycin-unreactive state to a puromycin-reactive state. At the same time, the A site of the 30S subunit remained filled. They concluded that the peptidyl-tRNA was in an A/P state, but one that differs from that observed in the hybrid

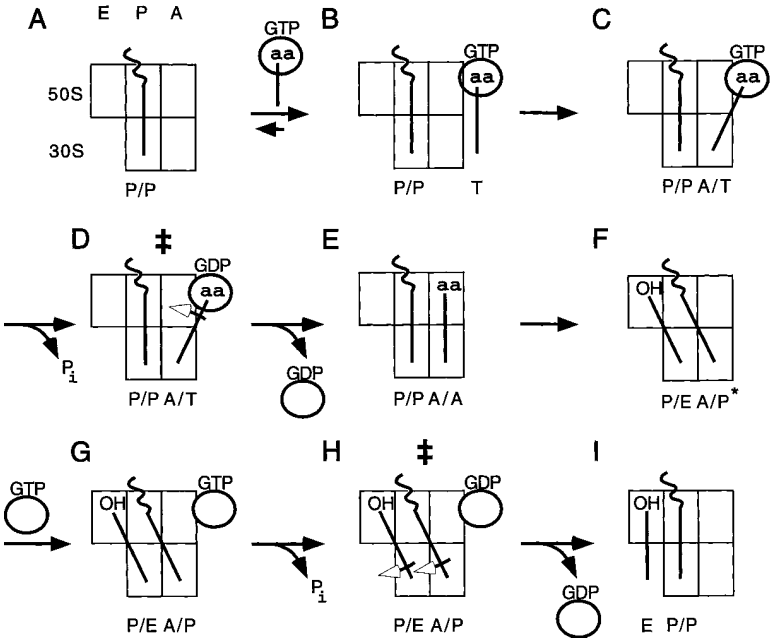

Figure 3. Elaborated Hybrid States Model, Incorporating Recent Findings that Establish Additional Intermediate States in the Translational Elongation Cycle

Symbols are as described in Figure 1. Open arrows emphasize the time resolution of factor binding from tRNA movement (Rodnina et al., 1995, 1997). Steps marked with the ‡ symbol are potential high-energy intermediates, corresponding to "unlocked" states of the ribosome-tRNA-mRNA complex (Spirin, 1985), as discussed in the text.

states experiments in that it is puromycin-reactive. Presumably, some GTP-independent activity of EF-G converts the state of peptidyl-tRNA in the 50S P site into one that is competent for the peptidyl transferase reaction. Since puromycin reactivity more rigorously characterizes the 50S P state of tRNA, we redefine the spontaneously derived A/P state as the "A/P*" state, to reflect its unreactivity toward puromycin, and the state observed in the EF-G deletion experiments as the A/P state.

In the last few years, these and other studies have provided evidence for the existence of additional intermediates in the elongation cycle, which are represented in an elaborated hybrid states model shown in Figure 3. First, the mechanism of EF-Tu-dependent aminoacyl-tRNA binding can be divided into several additional steps. Initial binding of the EF-Tu ternary complex to the ribosome (observed by fluorescent probes attached to the tRNA) is rapid and reversible and does not involve codon recognition (Figure 3B; Rodnina et al., 1995). This initial complex can form even if the A site is blocked with another aminoacyl-tRNA. Initial binding is followed by a series of conformational changes leading to codon recognition (Figure 3C) and GTP hydrolysis (Figure 3D; Rodnina et al., 1995). Upon dissociation of EF-TuGDP, the aminoacyl-tRNA enters the 50S A site (Figure 3E), leading to peptide bond formation (Figure 3F).

Until recently, binding of EF-G·GTP to the ribosome was thought to induce translocation, followed by GTP hydrolysis and release of EF-G·GDP from the ribosome (Kaziro, 1978). This conclusion was based principally on the observation that a single round of translocation could occur with nonhydrolyzable GTP analogs, while GTP hydrolysis was required for release of EF-G after translocation (Inoue et al., 1974), presumably serving to drive translocation in one direction (Spirin, 1985). However, recent pre-steady-state kinetic experiments show clearly that GTP hydrolysis occurs before translocation and accelerates translocation more than 50-fold relative to that observed with nonhydrolyzable GTP analogs (Rodnina et al., 1997). These new kinetic experiments require a reevaluation of the role of GTP hydrolysis in translocation, suggesting instead that a conformational transition in EF-G itself is in some way coupled to translocation. These experiments, together with the effects of the G domain deletion mutant mentioned above, suggest that EF-G-dependent translocation comprises at least four steps: (1) transition to a puromycin-reactive state soon after EF-G binding (Figure 3G); (2) GTP hydrolysis (Figure 3H); (3) translocation of the anticodon arms of the tRNAs; and (4) release of EF-G·GDP (Figure 3I).

This sequence of events is also supported by the observed effects of several antibiotics that block translocation at specific steps in the pathway outlined in Figure 3. Following binding of EF-G·GTP, thiostrepton inhibits the pathway at a step before GTP hydrolysis and translocation (Cundliffe, 1990). Viomycin allows GTP hydrolysis but prevents translocation (Modolell and Vazquez, 1977). Finally, fusidic acid allows GTP hydrolysis and translocation but prevents release of EF-G·GDP (Cundliffe, 1972).

Molecular Basis and Structural Background

Clearly, no precise description of the molecular mechanism of translocation will be possible until the structure of the ribosome is known at high resolution. Currently, we are at an intermediate stage of understanding ribosome structure. The structures of a dozen of the more than 50 ribosomal proteins have been solved to atomic resolution, and the three-dimensional positions of most of the proteins have been localized in the ribosome either by immuno-electron microscopy, neutron diffraction, or a combination of both (reviewed in Hill et al., 1990; Matheson et al., 1995). The secondary structures of the three ribosomal RNAs have been determined, mainly by comparative sequence analysis (reviewed in Noller, 1984); much information about their higher-order

folding has been obtained by cross-linking, footprinting, and directed hydroxyl radical probing studies (Brimacombe, 1991; Noller et al., 1995; Powers and Noller, 1995).

Direct structure determination of whole ribosomes or ribosomal subunits has been addressed both by crystallography and by electron microscopy reconstruction methods. Crystals of 70S ribosomes as well as 30S and 50S subunits have been obtained, most of which diffract to relatively low resolution. However, some crystals of 50S subunits diffract to better than 3 Å resolution (Yonath, 1992), raising hopes for an eventual atomic-resolution structure. This will depend on obtaining high-resolution phase information, a daunting problem for an asymmetric unit whose molecular weight approaches 2 MDa.

Meanwhile, electron microscopy reconstruction studies are providing low-resolution images of ribosomes and ribosome-tRNA complexes that have important general implications for the mechanism of translation. They reveal an extensive cavity between the two ribosomal subunits that has room enough to accommodate two or more tRNAs, as well as EF-G or EF-Tu (Frank et al., 1995; Stark et al., 1995). Indeed, these ligands are found to occupy the intersubunit space, according to difference maps obtained from ribosome-tRNA and ribosome·EF-Tu complexes (Agrawal et al., 1996; Stark et al., 1997a, 1997b). In the standard view of the ribosome structure, the tRNAs occupy the left-hand side of the intersubunit cavity, between the platform, cleft, and head of the small subunit and the L1 ridge and central protuberance of the large subunit (Figure 4), as was inferred previously from biochemical evidence (Wower et al., 1989; Noller et al., 1990).

Direct interpretation of structural models at the atomic level will not be possible until much higher-resolution structures are available. Meanwhile, identification and localization of specific structural features of ribosomal RNA and ribosomal proteins in low- and intermediate-resolution structural maps can be inferred from low-resolution structural models that have been deduced on the basis of extensive biochemical and physical constraints (Brimacombe et al., 1988; Stern et al., 1988; Malhotra and Harvey, 1994; Noller et al., 1995; Fink et al., 1996; Mueller and Brimacombe, 1997). Although the different models for the folding of 16S rRNA in the small ribosomal subunit differ in detail, there is general agreement about the locations of many structural features. Consensus among different research groups tends to be strongest for features for which there are the largest number of constraints and for which the fewest conflicting data exist. Most of these features are likely to be correctly placed, within the low resolution of the methods currently employed.

Assignment of density in electron microscopy reconstruction maps to individual specific tRNA molecules can be inferred from constraints obtained from numerous cross-linking, footprinting, and directed probing studies. In the view shown in Figure 4, the A-site (A/A state) tRNA is on the right and the P-site (P/P state) tRNA is on the left with their anticodon ends toward the viewer. Their acceptor ends bind to the peptidyl transferase center, located approximately in the middle of the interface surface of the large subunit; their anticodon ends interact with A and P codons in the decoding site, located in and around the cleft of the small subunit. The angle between the planes of the A- and P-site tRNAs has been determined by fluorescence energy transfer (Paulsen et al., 1983), difference electron microscopy reconstructions of ribosome·tRNA complexes (Agrawal et al., 1996; Stark et al., 1997a), and by localized hydroxyl radical probing between tRNA and tRNA analogs occupying the two sites (S. Joseph and H.N., unpublished data). There is general agreement between these approaches that the angle is on the order of 60°. The acceptor end of deacylated tRNA binds to the E site of the large subunit, near protein L1, which is located in the protuberance at the upper left corner of the large subunit. As discussed below, aminoacyl-tRNA is introduced at the right-hand side of the ribosome by EF-Tu. Accordingly, the tRNA progresses from right to left, from T to A to P to E states, as it transits the ribosome. The nascent polypeptide chain, which originates in the peptidyl transferase catalytic site, is believed to exit through channels leading from the interface side to the solvent side of the large subunit (Agrawal et al., 1996; Stark et al., 1997a).

The structures of EF-G and its complex with GDP have been solved by X-ray crystallography (Ævarsson et al., 1994; Czworkowski et al., 1994), as have the GTP and

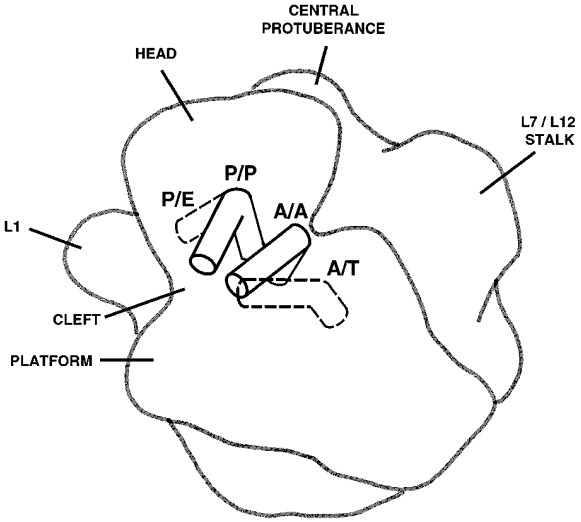

Figure 4. A Consensus Model Showing the Path of tRNA through the Ribosome

The positions of tRNA in different binding states (P/E, P/P, A/A, and A/T states) are based on the hybrid states model (Moazed and Noller, 1989b). tRNA positions are based on electron microscopy reconstruction studies (Agrawal et al., 1996; Stark et al., 1997a), fluorescence energy transfer experiments (Paulsen et al., 1983), chemical footprinting and cross-linking (reviewed in Brimacombe et al., 1995; Noller et al., 1995), and directed hydroxyl radical probing (Joseph et al., 1997; see also Figure 7). The view is from the solvent face of the 30S subunit. The anticodon ends of the tRNAs are oriented toward the viewer, interacting with the cleft and neck regions of the 30S subunit. The acceptor ends of the A/A and P/P state tRNAs interact with the peptidyl tranferase region in the middle of the 30S face of the 50S subunit. This arrangement is similar to models proposed earlier, based mainly on biochemical constraints (Wower et al., 1989; Noller et al., 1990).

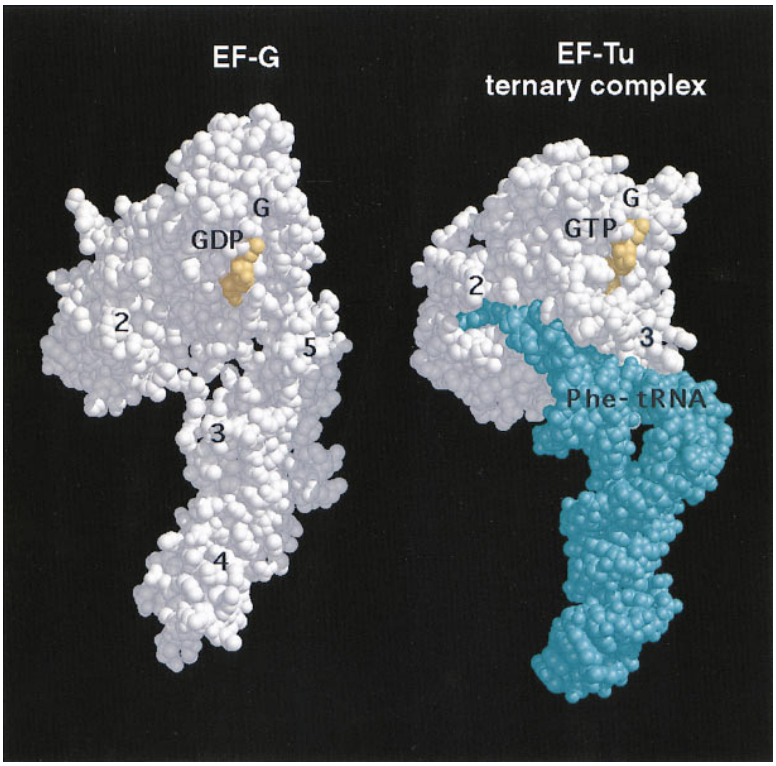

Figure 5. Structures of EF-G and the EF-Tu Ternary Complex

Shown here are the crystal structures of the EF-G·GDP complex (Czworkowski et al., 1994) and the EF-Tu·Phe-tRNA·GTP ternary complex (Nissen et al., 1995). The crystal structure of free EF-G has also been determined (Ævarsson et al., 1994) and has a conformation similar to that of EF-G·GDP.

GDP forms of EF-Tu and its ternary complex with aminoacyl-tRNA and GTP (Berchtold et al., 1993; Nissen et al., 1995). Most remarkably, EF-G is strikingly similar in its overall shape to the ternary complex of EF-Tu·Phe-tRNA·GTP (Figure 5). Not only are the first two domains of EF-Tu and EF-G closely similar to each other, but the latter three domains of EF-G (domains 3, 4, and 5) appear to mimic the structure of tRNA in the EF-Tu ternary complex (the acceptor arm, anticodon arm, and elbow of tRNA, respectively). This remarkable example of apparent molecular mimicry implies an underlying functional similarity between the catalytic mechanisms of EF-Tu and EF-G.

Interactions of the Factors with the Ribosome

For many years, it has been known that EF-G and EF-Tu compete for a common binding site on the ribosome (Richman and Bodley, 1972; Moazed et al., 1988). The recent crystal structures described above reinforce the view that a common element of the ribosome activates the GTPase activity of both factors. A strong candidate for the GTPase activator is the sarcin loop in domain VI of 23S rRNA, which interacts with both factors (Hausner et al., 1987; Moazed et al., 1988). Two cytotoxins (sarcin and ricin) modify this loop and specifically inactivate the ribosome-dependent GTPase activity of EF-G (Fernandez-Puentes and Vazquez, 1977; Endo and Wool, 1982), and recent experiments show that EF-G binds specifically to the sarcin loop alone (Munishkin and Wool, 1997). In addition, in vitro studies suggest that GTP hydrolysis is regulated by the stalk of the ribosome, a protein-rich extension of the large subunit composed of proteins L7/L12 and L10, and an element of domain II of 23S rRNA that is believed to be conformationally mobile (Traut et al., 1995).

Recently, the position and orientation of EF-G in the ribosome has been mapped by site-directed hydroxyl radical probing using Fe(II)-EDTA tethered to 18 different sites on the surface of EF-G (Wilson and Noller, 1998). These data provide proximity relationships between individual amino acid positions on the crystallographically determined structure of EF-G and specific nucleotide positions in 16S and 23S rRNA. These data provide a set of constraints that are sufficient to position EF-G in the ribosome at low resolution. The factor is located between the two ribosomal subunits, in the intersubunit space. Its G domain (domain 1) faces the large subunit, near the sarcin loop and rRNA elements of the stalk, while domain 2 faces the small subunit, near the binding site of protein S4. Domain 4, which appears to mimic the anticodon arm of tRNA of the ternary complex, is directed toward the decoding center with its tip positioned close to several rRNA elements of the small subunit tRNA sites.

This position and orientation for EF-G in the ribosome is very similar to that observed for the EF-Tu ternary complex, determined independently by electron microscopy reconstruction methods (Stark et al., 1997b; and see below). The G domain of EF-Tu rests against the base of the stalk of the large subunit and domain 2 faces the small subunit similar to orientations predicted for the corresponding domains of EF-G. The anticodon arm of the bound tRNA is directed into the decoding site, as is expected. These findings clearly explain the observed competition between EF-G and EF-Tu for their interaction with the ribosome.

Minimal Requirements for Translocation

Important insights into the nature of the translocation mechanism have come from in vitro studies with simplified model systems. The discovery of factor-independent translocation (Pestka, 1969; Gavrilova and Spirin, 1971) established that it is fundamentally a ribosomal mechanism, as already mentioned. Equally significant is the finding that translocation can take place in the absence of a messenger RNA. Under certain in vitro conditions, lysyl-tRNA can be polymerized by the ribosome into polylysine (Belitsina et al., 1981). These experiments provided evidence for a possibility that had been suspected previously—that the translocation machine acts directly on tRNA and that movement of mRNA is passive, driven by its association with tRNA. A further requirement is that there must be a tRNA in both the A and P sites for translocation of a P-site tRNA (Lucas-Lenard and Haenni, 1969). However, even a simple anticodon stem-loop in the 30S A site is sufficient for EF-G-dependent translocation of both tRNAs, as discussed below (S. Joseph and H. N., unpublished data). Finally, it is important to keep in mind that the tRNAs interact not only with mRNA, via their codon–anticodon interactions, but also with the ribosome itself (Nirenberg and Leder, 1964; Rose et al., 1983; Moazed and Noller, 1986, 1989a; Samaha et al., 1995; von Ahsen and Noller, 1995). Clearly, these tRNA-ribosome interactions need to be disrupted and reformed before and after (and perhaps during) translocation of tRNA.

Indications of the strengths of the interactions and the energies involved in translocation come from binding and kinetic studies. Factor-independent translocation is much slower than EF-G-catalyzed translocation. The activation energy for spontaneous translocation is around 20 kcal/mol while that for EF-G-catalyzed translocation is about 7 kcal/mol (Schilling et al., 1992). EF-G thus acts as a true catalyst, somehow coupling the energy of GTP hydrolysis to movement. (For comparison, the free energy of ATP hydrolysis, which must be very similar to that of GTP hydrolysis, under physiological conditions is estimated to be about 12 kcal/mol; Rosing and Slater, 1972.) Thus, the difference in the activation energies between spontaneous and EF-G-catalyzed translocation can be accounted for by the free energy of GTP hydrolysis. However, part of the catalytic effect of EF-G must be due to binding of EF-G·GTP to the ribosome, which leads to a substantial catalysis of translocation in the absence of GTP hydrolysis (Rodnina et al., 1997). In movement of tRNA from the pretranslocation state to the posttranslocation state, what is the structural correlate of this thermodynamic barrier? We know that one barrier to be broken is the collective interaction of peptidyl tRNA (A/P state), deacylated tRNA (P/E state), and mRNA, with the ribosome, although we do not know whether this barrier is rate limiting for translocation. The sum of the free energies of these binding interactions is estimated to be of the order of 20 kcal/mol. However, it seems quite unlikely and unnecessary that these interactions would all be broken simultaneously, leaving the only marginally stable ribosome-independent codon–anticodon interaction of peptidyl-tRNA with mRNA to maintain the translational reading frame. A more likely possibility is that the various ribosomal interactions are broken and reestablished in some step-wise fashion, implying the existence of further intermediate states of tRNA binding that are yet to be observed.

Two Basic Problems of Translocation

In catalyzing translocation, EF-G surmounts two fundamental problems. First, a significant activation energy barrier must be overcome, most likely involving disruption of tRNA-ribosome (and possibly mRNA-ribosome) interactions. Second, these disruptions, as well as movement of tRNAs and messages, must be accomplished without loss of the translational reading frame. In other words, the codon–anticodon interactions must be steadfastly maintained, even though there must be at least transient disruption of tRNA-ribosome interactions, the very interactions that are believed to allow ribosomal stabilization of the inherently weak binding between codon and anticodon. These two problems are the opposing ones of speed versus accuracy that are often encountered in biological systems. How the ribosome deals with this paradox is a mystery that has been the subject of much wonder and speculation.

Locking and Unlocking: Ribosomal Switches

It has been proposed that the barrier to movement of tRNA is caused by "locking" of the tRNA-ribosome complex in a fixed conformation (Spirin, 1985). The locked state can be considered to be a kinetic trap that is imposed on the complex by the aforementioned activation barrier. According to this view, EF-G has been suggested to catalyze translocation by "unlocking" the ribosome in a GTP-dependent manner, overcoming the activation energy barrier for tRNA movement. There may be more than one locked state, defined not only by the conformation of the ribosome, but also by the binding states of the tRNAs. The transition between the pre- and posttranslocational states would involve an unlocked state, during which movement could occur. Physically, unlocking could include disruption or rearrangement of the tRNA-ribosome interactions discussed above as well as rearrangement of intramolecular interactions within the ribosome itself. There is mounting evidence for the occurrence of conformational switching in the ribosome. Such switches may relate to movement associated with elongation factor–dependent events in two different ways: (1) unlocking of the tRNA-ribosome complex to allow translocation to proceed, and (2) the movement associated with translocation itself.

The idea that perturbations of ribosome structure can cause it to tighten or loosen its grip on tRNA originated with the discovery of mutations in ribosomal proteins that affect translational accuracy. Streptomycin-resistant or -dependent alleles of protein S12 were found to have hyperaccurate (restrictive) phenotypes (Gorini, 1971). Suppressor mutations of streptomycin dependence were mapped to proteins S4 and S5, which were found to have increased translational error frequencies (*ram*, or ribosomal ambiguity phenotypes) when segregated from the streptomycin-dependent backgrounds. It has been found that restrictive ribosomes have generally lower affinity, while *ram* ribosomes have higher affinity, for aminoacyl tRNA (Karimi and Ehrenberg, 1994),

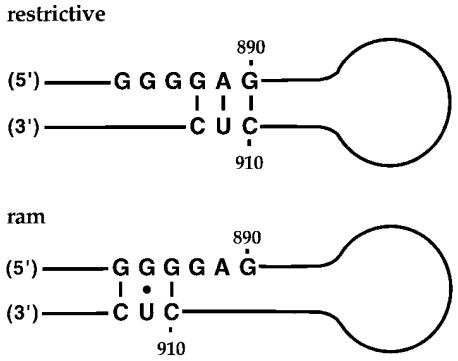

Figure 6. The Triplet Switch

Lodmell and Dahlberg (1997) have shown that there are two alternative biologically essential pairings for this region of 16S rRNA. Mutations that favor the upper pairing confer hyperaccurate (restrictive) translational accuracy phenotypes; mutations that favor the lower pairing confer error-prone (*ram*) phenotypes.

although this does not appear to fully account for their differences in tRNA selectivity. Intriguingly, the affinity of these ribosomes for peptidyl-tRNA appears to be inversely correlated to their affinity for aminoacyl-tRNA; peptidyl-tRNA dissociates more readily from *ram* ribosomes and binds more tightly to restrictive ribosomes (Karimi and Ehrenberg, 1996). These results suggest that the affinities of the ribosomal binding sites for tRNA are finely balanced and regulated by certain ribosomal proteins.

Mutations in 16S rRNA can also confer streptomycin-resistant and *ram* phenotypes. One set of mutations involves a pseudoknot in 16S rRNA that is stabilized by assembly of protein S12 (Powers and Noller, 1994). S4 *ram* mutations have been observed to affect the local conformation around specific locations in 16S rRNA, and, conversely, mutations in 16S rRNA can confer *ram* phenotypes (Allen and Noller, 1989, 1991). Such results suggest to RNA centrists that mutations in ribosomal proteins could exert their effects via perturbation of the conformation of 16S rRNA and that regulation of the binding of tRNA could be based fundamentally on rRNA mechanisms. Since translocation must involve changes in the mode of interaction of the ribosome with tRNA, an RNA-based mechanism is attractive, not only because of the inherent flexibility of RNA but also in the simple way by which alternative modes of intramolecular base pairing could precisely define different binding states.

Recent studies of a series of localized mutations in 16S rRNA suggest that just such a mechanism may, in fact, play a role in the translational mechanism (Lodmell and Dahlberg, 1997). These studies show that there are two alternative base pairings for the three–base pair sequence (910) CUC (912) of 16S rRNA, both of which are required for ribosome function (Figure 6). The CUC sequence pairs either with (888) GAG (890) or with the immediately adjacent (885) GGG (887) sequence. Mutations favoring the 888 pairing have restrictive phenotypes, while those favoring the 885 pairing confer *ram* phenotypes and are able to suppress S5 *ram* mutations and S12 restrictive mutations, respectively. Both S5 and S12 protect bases in this region of the RNA during assembly (Stern et al., 1989), suggesting that regulation of translational accuracy by these proteins may involve modulation of this RNA conformational switch. Interestingly, the reactivity of the adjacent base A908 was shown to correlate with the phenotypes of mutations in proteins S4 and S12 in earlier studies (Allen and Noller, 1989). In addition, binding of tRNA to the 30S A site was found to cause enhanced reactivity of another nearby base, A892, indicative of a tRNA-dependent conformational change in this region of 16S rRNA (Moazed and Noller, 1986). Although these findings imply that the triplet switch is primarily involved in tRNA selection, there are hints that it may also be related to the process of translocation. First, mutations that stabilize the 888 pairing are hypersensitive to spectinomycin, an inhibitor of EF-G-dependent translocation (Lodmell and Dahlberg, 1997). Second, although these same mutations have a restrictive phenotype, they unexpectedly show an elevated level of translational frameshifting, a potential consequence of impaired translocation. Finally, the nature of the switch itself—movement of the CUC sequence by precisely three nucleotides between adjacent complementary triplets—bears an intriguing resemblance to the process of translocation.

Another potential switch was suggested by results of chemical probing studies on tRNA-ribosome complexes (Moazed and Noller, 1989b). A set of six bases (called class III sites) in 16S rRNA was observed to be protected not only by binding of tRNA to 30S subunits, but also by binding of certain antibiotics or 50S subunits to 30S subunits, in the absence of tRNA. Since it is well known that all three kinds of ligands can bind simultaneously to 30S subunits (and, in fact, bind cooperatively), it is unlikely that the six bases are protected by direct contact with the ligands; more likely, their protection is a result of a conformational change in the 30S subunit that can be stabilized by binding of tRNA, 50S subunits, or antibiotics. The class III sites can be further subdivided according to their protection by antibiotics. One subset, bases A790, G791, and A1394, is protected by edeine, a P-site inhibitor; the other subset, bases A909, A1413, and G1487, is protected by streptomycin, an A-site-directed drug. All six are protected by aminoglycoside antibiotics, such as neomycin. Intriguingly, one of the class III sites, A909, is immediately adjacent to the CUC sequence involved in the triplet switch.

Moving Parts of the Engine

As discussed above, the movement associated with translocation is somehow inherent in the ribosome itself, along with its bound tRNAs and their associated mRNA. It is hard to escape the conclusion that one or more of these components must undergo machine-like movement during the elongation cycle. Investigators studying the process of translocation have long searched for "moving parts"; we next consider the likely candidates.

Protein L7/L12 is closely associated with the functions of the elongation factors (Möller, 1990; Traut et al., 1995). It has been suggested that ribosomal movement during the elongation cycle could involve conformational rearrangement of L7/L12, which contains a flexible hinge

joining its globular N- and C-terminal domains (Liljas and Gudkov, 1987; Traut et al., 1995). This protein is present in four copies per ribosome, most likely as a pair of dimers, and is required for binding of elongation factors to the ribosome, which is believed to be through its C-terminal domain (Girshovich et al., 1981). It is visible in some electron micrographs as an extended stalk at the right-hand side of the large subunit, but immunoelectron microscopy and cross-linking studies have also placed its C-terminal domain at an alternative position at the base of the stalk near protein L11 and at a third position below the left side of the central protuberance of the subunit, near the position of protein L2 (Traut et al., 1983; Olson et al., 1986). The site near L11 is the most likely position for its interaction with the elongation factors. Movement of the C-terminal domain between one or more of these three positions would provide the proposed movement (Traut et al., 1995). These locations for the L7/L12 C-terminal domain are all relatively remote from the positions of tRNA, however. If movement of L7/L12 is involved in translocation of tRNA, it would seem to exert its influence indirectly in some way.

The fact that all ribosomes are composed of two subunits suggested many years ago that translocation may somehow be coupled to relative movement between the subunits (Bretscher, 1968; Spirin, 1968). The main conclusion from the hybrid states studies—that translocation of the two extremities of tRNA can occur independently with respect to the two subunits—further reinforces the suggestion that this could be the case (Moazed and Noller, 1989b). Such movement could, in principle, involve the subunits themselves or smaller domains or subdomains of the subunits. At the other extreme is the proposal that movement involves global rearrangement of the structure of the ribosome, as suggested by the results of electron tomography experiments (Öfverstedt et al., 1994).

Involvement of the Small Subunit in tRNA Movement

A further clue to the nature of the translocation mechanism comes from consideration of the role of the small ribosomal subunit. Although the site of action of EF-G has traditionally been identified with the large subunit, the movement of tRNA depends critically on the small subunit. An early indication was the identification of mutations in protein S5 as well as in 16S rRNA that confer resistance to spectinomycin, a drug that inhibits EF-G-dependent translocation (Bollen et al., 1968; Sigmund et al., 1984). Second, as the hybrid states experiments show, the strictly EF-G-dependent step of translocation involves movement of the anticodon ends of the tRNAs relative to the small subunit (Figure 2), even though both subunits are required for movement to occur. There is also a requirement for occupation of the small subunit A site by tRNA; since A-site occupancy requires P-site occupancy, a tRNA is also required in the P site. Recently, the boundaries of the A-site requirement have been tested systematically, using oligonucleotide analogs of tRNA (S. Joseph and H. N., unpublished data). It was found that ribosome complexes in which the A-site tRNA is replaced by a minimal four–base pair anticodon stem-loop analog, containing only 15 nucleotides, undergo efficient EF-G-dependent translocation. Both the oligonucleotide and the full-length P-site tRNA are translocated, as is the mRNA. This result shows that neither the elbow region nor the acceptor arm of A-site tRNA are required for translocation. Since it is well established that the anticodon stem-loop of P-site tRNA interacts exclusively with the small subunit of the ribosome (Rose et al., 1983; Moazed and Noller, 1986; Hüttenhofer and Noller, 1992), it can be concluded that the EF-G-dependent step of translocation of an aminoacyl-tRNA from the A site to the P site can be reduced to rearrangement of the interactions between its anticodon stem-loop and the 30S subunit.

It is generally accepted that P-site codon–anticodon interaction is important for maintaining the correct translational reading frame. However, following translocation, this interaction is disrupted, since it is believed that there is no significant codon–anticodon interaction in the E site (but see Nierhaus, 1990). Therefore, during translocation, as the P-site codon–anticodon interaction is disrupted, the A-site tRNA must take over this role. Moreover, its codon–anticodon pairing must be stably maintained during its movement from A site to P site. This pairing is significantly more stable in the ribosome than in solution (Grosjean et al., 1976). Thus, whatever aspect of ribosomal structure is responsible for stabilizing codon–anticodon interaction must accommodate the movement. For example, if stabilization is promoted by an environment that excludes water, this environment would have to be maintained around the codon–anticodon pair throughout the entire path ($>$20 Å) of its intraribosomal excursion. If pairing is stabilized by interactions with structural elements of the ribosome, such interactions would have to be maintained throughout translocation.

Based on this reasoning, a key focus of the translocation mechanism can be considered to be movement of the A-site anticodon stem-loop with respect to the 30S subunit and stabilization of the interaction with its associated codon. At the start of translocation, a tRNA also occupies the 30S P site, which probably dissociates from its codon and from the 30S subunit during translocation, and so would seem to be of diminishing importance to the ribosome. In contrast to A-site tRNA, a full-length P-site tRNA is required for EF-G-dependent translocation (S. Joseph and H. N., unpublished data). This requirement suggests that interactions between the deacylated tRNA and the 50S E site are in some way crucial for translocation. At the end of the translocation event, a single tRNA anticodon occupies the 30S subunit, which is alone responsible for maintaining the reading frame. What remains to be understood is what happens between these two states. At some point, the A-site interactions are disrupted, and, at some point, the P-site interactions are formed. These two processes could overlap in time, in a concerted reaction, in which new interactions are formed simultaneously as the old ones are disrupted. Or, some of the P-site interactions could form prior to disruption of the A-site interactions. A further possibility is that another class of interactions, neither A nor P in the usual sense, is transiently formed to promote stabilization of tRNA binding and tRNA-mRNA

interactions during translocation. There is no strong reason to exclude any of these possibilities from the available evidence. Nierhaus and colleagues have proposed a kind of moving frame of reference in which the tRNAs are transported within the ribosome, which could be considered as yet a fourth possibility (Nierhaus, 1990).

From the above discussion, it seems likely that a primary source of ribosomal dynamics originates within the small subunit. The small subunit has often been considered to be a flexible structure, virtually two dimensional by comparison with the large subunit; no intradomain RNA-RNA tertiary interactions have been identified in 16S rRNA, in spite of extensive attempts to find them. Both its head and platform features are largely autonomous structures, attached to the main body of the subunit by only minimal connections. The head of the subunit, which contains a 400-nucleotide domain of 16S rRNA and eight proteins, is connected to the body of the ribosome by as little as a single strand of RNA (Brimacombe et al., 1988; Stern et al., 1988; Mueller and Brimacombe, 1997). Such features are highly suggestive of independent movement. Moreover, the site of convergence of the body, head, and platform (which correspond roughly to the 5′, 3′ major, and central plus 3′ minor domains of 16S rRNA and their associated proteins, respectively) is at the cleft and neck of the subunit, precisely where the anticodon arms of the two tRNAs have been localized (Agrawal et al., 1996; Stark et al., 1997a). If movement of tRNA were coordinated with movement of one or more of these structural elements of the small subunit, translocation could be accomplished while transiently maintaining intermolecular contacts between the mRNA-tRNA complex and the 30S subunit.

The Catalytic Role of EF-G

Given the important role of the A-site tRNA anticodon and its interactions with the small subunit for translocation, the observed mimicry of the anticodon arm by domain 4 of EF-G is all the more intriguing. Directed probing experiments show that in fusidic acid-stabilized EF-G·GDP·ribosome complexes, domain 4 is indeed positioned at or very near the site of the A-site tRNA anticodon arm (Wilson and Noller, 1998). This is illustrated in Figure 7, which shows the relative positions of tRNA, EF-G, and the EF-Tu·tRNA ternary complex docked on a model for the 30S subunit, using constraints obtained from cross-linking, footprinting, and directed hydroxyl radical probing. The EF-Tu ternary complex was positioned by superimposing its G domain on that of the docked EF-G; its resulting position and orientation are remarkably similar to that obtained directly by recent electron microsopy reconstruction studies (Stark et al., 1997b).

A potential role for domain 4 can be inferred from the effects of deleting it from the structure of EF-G. Kinetic studies show that this results in a 1000-fold reduction in the rate of elongation, without affecting either the binding of EF-G to the ribosome or its GTPase activity. This suggests that domain 4 is in some way important for coupling of GTP hydrolysis to movement of tRNA. Since it is known from nonenzymatic translocation experiments that the ability to achieve movement of tRNA is inherent in the ribosome, it seems unlikely that domain 4 itself physically moves the tRNA. A more likely possibility is that its interactions with structural features of the 30S subunit trigger the movement—i.e., that it promotes a structural rearrangement that allows the ribosome to overcome the free energy barrier to translocation. This transition would correspond to the "unlocking" event discussed above. The proposed structural rearrangement could then lead to transient stabilization of the A-site codon–anticodon interaction, which could be coupled to the movement itself.

A Unifying Mechanism for EF-G and EF-Tu

A possible clue to the nature of tRNA movement comes from the relative positions of the A- and P-site (A/A and A/P state) tRNAs, and the EF-Tu-bound tRNA (A/T state tRNA) in the ribosome, whose acceptor end lies far from its eventual position in the A/A state. Although the positions shown in Figure 7 come from modeling, extensive experimental constraints support the positions of the three tRNAs approximately as shown. Furthermore, the predicted placements agree closely with the positions of density for tRNA and the EF-Tu ternary complex observed at low resolution using electron microscopy reconstruction methods (Agrawal et al., 1996; Stark et al., 1997a, 1997b). The A/T and A/A tRNAs (Figure 7D) differ in position by two rotational movements. First is a rotation of about 60° of the plane of the A/T tRNA around an axis that is normal to its anticodon arm and centered near its anticodon (the [a] axis; Figure 7D). Second is a rotation of about 60° around an axis that is coaxial with its anticodon arm (axis [b]). These two rotations result in movement of the A/T tRNA to the A/A position. Interestingly, the overall process of translocation, movement of tRNA between the A/A and P/P states, can be accomplished by two closely related rotational movements: an ~60° rotation of the A/A tRNA around the (a) axis, followed by an ~60° rotation around its anticodon arm axis (the A/A equivalent of the [b] axis). The main difference is that the second ([b] axis) 60° rotation is in the opposite sense of that for the A/T to A/A transition. Finally, a transition from the P/P state to the P/E state (not shown) could be accomplished by another 60° rotation around the anticodon axis of the P/P tRNA, this time in the same sense as the (b) axis rotation of the A/T to A/A transition (see Figure 4).

The origin of the putative rotational movements is not known. However, since both rotational axes are centered on the small subunit, rearrangement of its structure seems a strong possibility. An obvious potential mechanism for the (a) axis rotation is turning of the head of the small subunit. The (a) axis is, within the uncertainty of current structural information, approximately coaxial with the neck of the subunit, whose RNA structural correlate is the 926 region stem of 16S rRNA. Rotation about the (a) axis could involve this RNA helical element in some way. Candidates for the basis of the (b) axis rotation are less obvious, but might be accomplished by nodding of the head toward and away from the body. In support of this model, neutron scattering experiments have provided evidence that the principle intraribosomal structural movement that occurs during the pre- to post-translocational transition involves the head of the small subunit (Serdyuk et al., 1992).

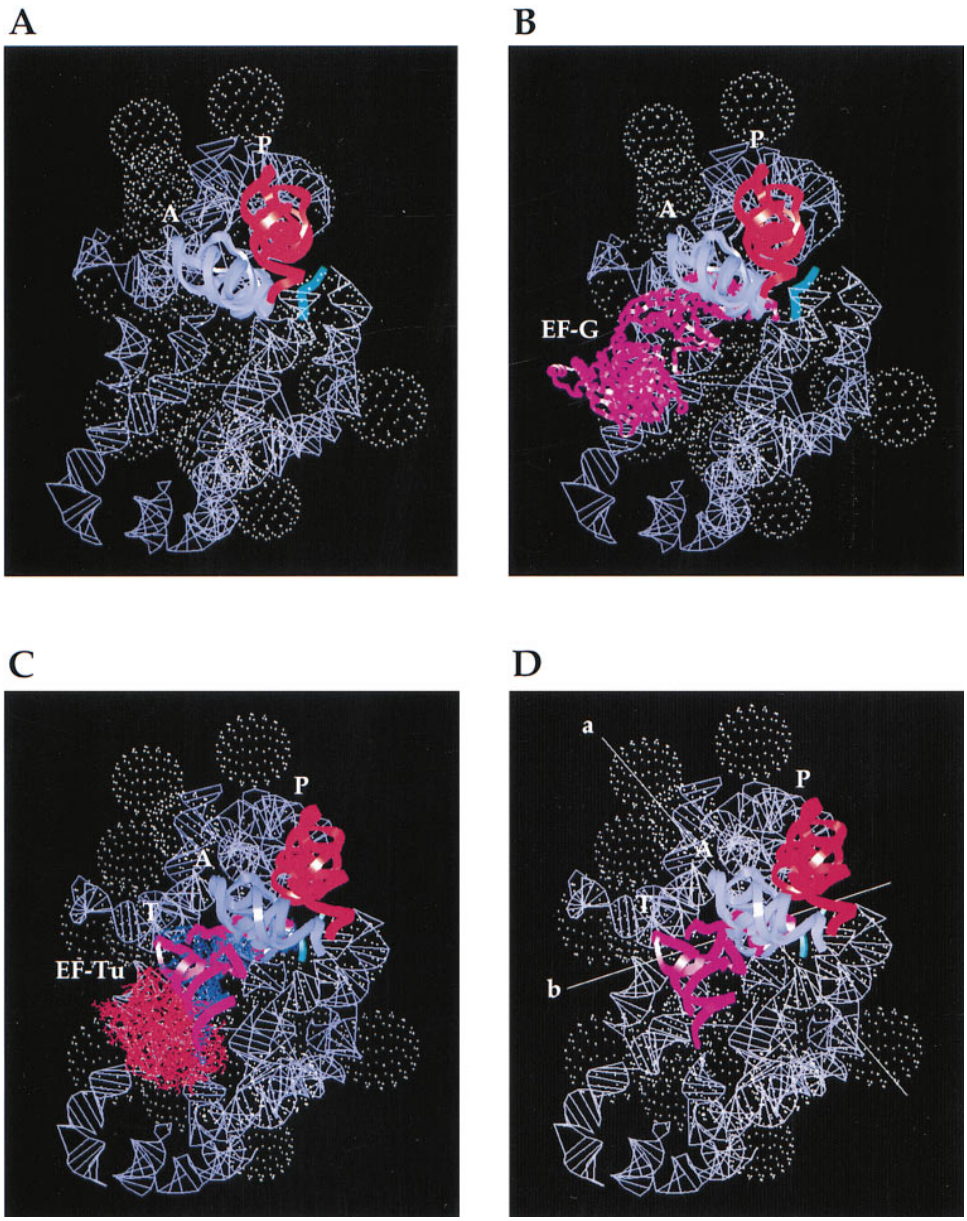

Figure 7. Approximate Positions of tRNA in Different Ribosomal Binding States, and the Positions of Elongation Factors EF-G and EF-Tu Relative to a Model for the 30S Ribosomal Subunit, Viewed from the 50S Interface Side

The tRNA states are designated A (A/A state, light blue), P (P/P state, red) and T (A/T state, magenta), respectively. EF-G is colored magenta and EF-Tu red. In the 30S model, the positions of the proteins from the neutron map (Capel et al., 1987) are shown as dotted spheres, and 16S rRNA as light blue lines. The A and P site mRNA codons are shown in cyan.

(A) Two tRNAs in the A/A and P/P states, docked according to constraints obtained mainly from chemical footprinting (Moazed and Noller, 1986) and directed hydroxyl radical probing (Joseph et al., 1997). The letters are near the tRNA elbows, and the acceptor ends are at the lower right of the tRNAs, oriented toward the viewer.

(B) Docking of the crystal structure of EF-G·GDP (Czworkowski et al., 1994) on the 30S model, showing its position relative to that of the tRNAs. The position and orientation of EF-G were determined by directed hydroxyl radical probing of rRNA in the ribosome from 18 positions on the surface of EF-G (Wilson and Noller, 1998). The globular domain at the lower left is the G domain. Domain 4 reaches up to the right into the neck region at the base of the cleft, overlapping the position of the anticodon arm of the A/A tRNA. Note that the tRNA in the A/A state would not coexist with bound EF-G, which was mapped in the posttranslocational state.

(C) Position of the EF-Tu·tRNA·GTP ternary complex, obtained by superimposing the G domain of its crystal structure (Nissen et al., 1995) on that of EF-G. The anticodon arm of EF-Tu-bound tRNA follows a similar path to that of domain 4 of EF-G, overlapping with the anticodon region of the A/A tRNA. The A/A tRNA would be absent during binding of the ternary complex.

(D) Positions of tRNA in the A/T, A/A, and P/P states. The planes of the three tRNAs are related by a rotation of approximately 60° around the (a) axis, plus a second rotation around the axis of the anticodon arm (labeled axis [b], in the case of A/T tRNA), also of about 60°.

Finally, this model suggests a unifying mechanism for elongation factors EF-G and EF-Tu, whose structural similarity (Figure 5) is striking, yet baffling. Superficially, these factors appear to be involved in translational steps that seem mechanistically unrelated; EF-G catalyzes translocation, while EF-Tu introduces aminoacyl-tRNA to the ribosome. Their fundamental mechanisms, however, may be to catalyze the two very similar sets of rotational steps, which may themselves be innate properties of ribosomal mechanics. The main differences between the two processes would be the different initial states of the tRNAs undergoing movement in each case (A/T versus A/A) and the opposite sense of the (b) axis rotation for the two kinds of movement. Such differences could be accounted for by different detailed interactions between the factors and the ribosome, which could couple the same fundamental mechanism to movement of the two different tRNAs. Most provocative is the structural similarity between domain 4 of EF-G and the anticodon of tRNA in the EF-Tu ternary complex. This could be related to the proposed underlying similarity in their mechanisms. In the case of EF-G, domain 4 may coordinate or trigger conformational changes leading to translocational movement from A to P site. In the ternary complex, interactions between the anticodon arm of tRNA and the ribosome could accomplish the analogous task, leading to movement of the EF-Tu-bound aminoacyl-tRNA into its fully ribosomal position. Such a mechanism would account for the RNA mimicry observed in the structure of EF-G and further suggests that the primeval translocase could itself have been made of RNA—tRNA, for example.

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